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# Piceatannol, a natural hydroxylated analog of resveratrol, promotes nitric oxide release through phosphorylation of endothelial nitric oxide synthase in human endothelial cells

Y. SON, J.H. LEE, Y.-K. CHEONG, H.C. JUNG, S.-O. JEONG[1], S.H. PARK[2], H.-O. PAE[1,2]

Department of Anesthesiology and Pain Medicine, Wonkwang University School of Medicine, Iksan, South Korea
[1]Department of Microbiology and Immunology, Wonkwang University School of Medicine, Iksan, South Korea
[2]Institute for Metabolic Disease, Wonkwang University, Iksan, South Korea

**Abstract. – OBJECTIVE: Endothelial dysfunction associated with many cardiovascular diseases is largely due to reduced nitric oxide (NO) derived from endothelial NO synthase (eNOS). Piceatannol (trans-3,4,3',5'-tetrahydroxystilbene; Pic) is reported to have cardiovascular therapeutic effects. However, the cellular and molecular mechanisms underlying the cardioprotective effects of Pic are still unclear. Here, we investigated whether Pic could influence endothelial NO release in human umbilical vein endothelial cells (HUVECs).**

**MATERIALS AND METHODS: In HUVECs exposed to Pic, NO production and phosphorylation of eNOS and protein kinase B (Akt) were determined by using a commercially available NO assay kit and Western blot analysis, respectively.**

**RESULTS: Pic stimulated dose- and time-dependent NO production via eNOS phosphorylation. Pic also stimulated dose-dependent phosphorylation of Akt. Interestingly, NO production and eNOS phosphorylation in response to Pic were significantly abolished by the phosphoinositide 3-kinase (PI3K)/Akt inhibitor LY294002.**

**CONCLUSIONS: Pic is capable of inducing eNOS phosphorylation and the subsequent NO release, presumably, by activating PI3K/Akt pathway. The potential efficacy of Pic, a natural hydroxylated analog and a metabolite of resveratrol, may aid in the prevention of cardiovascular diseases characterized by endothelial dysfunction.**

*Key Words:*
Piceatannol, Nitric oxide, Endothelial nitric oxide synthase, Endothelial cells, Endothelial dysfunction.

## Introduction

Endothelial dysfunction, which is characterized by reduction of the bioavailability of vasodilators, particularly endothelial nitric oxide (NO), and/or an increase in endothelium-derived contracting factors, has been implicated in numerous cardiovascular diseases, such as hypertension, coronary artery disease, and chronic heart failure[1,2]. The free radical NO, which is synthesized endogenously from *L*-arginine by the action of the enzyme NO synthase (NOS), has a wide range of biological properties[3]. In endothelial cells, NO is produced mainly by the action of endothelial NOS (eNOS). At least six eNOS phosphorylation sites have been identified so far; however, major changes in eNOS function have been reported for the phosphorylation of serine 1177 (for activation) and threonine 495 (for inhibition) in the human eNOS sequence[4]. The eNOS phosphorylation has been shown to be regulated by a complex series of regulatory mechanisms. A number of kinases have been reported to phosphorylate eNOS and increase NO production. Some of them, such as protein kinase A, are activated by an increase in intracellular calcium ion, whereas kinases, such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), can be activated independently of an increase in intracellular calcium ion[5-9].

Because of the important role of NO in endothelial function[10-16], abnormalities in endothelial NO production are thought to contribute to the pathogenesis of many cardiovascular disorders, such as those of atherosclerosis and hypertension[17]. There is a growing body of evidence that some naturally occurring polyphenolic compounds derived from dietary sources or from specific medicinal plants may positively influence endothelial NO production and, thus, promote endothelial function[1]. The identification of such compounds and better understanding of their

*Corresponding Author:* Hyun-Ock Pae, Ph.D; e-mail: hopae@wku.ac.kr

molecular mechanisms of action may provide valuable strategies for the prevention of cardiovascular diseases.

Piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene; Pic) is a naturally occurring polyphenolic compound found in various kinds of food[18,19], such as sugar cane, berries, passion fruit seeds, peanuts, grapes, wine, and white tea. It is a natural hydroxylated analog of resveratrol (*trans*-3,5,4′-trihydroxystilbene), a well-known active ingredient in red wine[20-22], and also a metabolite of resveratrol[21]. Because of their structural similarity, Pic and resveratrol are believed to possess very similar pharmacologic properties[23]. In fact, both Pic and resveratrol have been reported to have cardioprotective activities[24,25].

Based on the extensive data favoring an effect of Pic on the vasculature[25] and the central role of endothelial NO in vascular function[8-16], we hypothesized that Pic would influence endothelial NO release from cultured human umbilical vein endothelial cells (HUVECs). To test this hypothesis, we investigated the molecular mode of action of Pic and characterized a possible upstream signaling pathway that leads to NO production.

## Materials and Methods

### Reagents and Antibodies

Pic, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin, Hank's balanced salt solution (HBSS), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 and $N^G$-nitro-*L*-arginine methyl ester (L-NAME) were purchased from Calbiochem (La Jolla, CA, USA). The horseradish peroxidase (HRP)-conjugated second antibodies to rabbit and mouse IgG and the primary antibodies to phosphor (p)-eNOS, eNOS, p-Akt and Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). Actin antibody and other reagents used for molecular studies were obtained from Sigma-Aldrich. Stock solutions of Pic at 100 mM in DMSO were stored at –80°C before use.

### Cell Culture

Primary HUVECs were purchased from Cascade Biologics Inc. (Portland, OR, USA) and used between the passage 3 and 8. HUVECs were grown in EGM-2 medium (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), streptomycin (100 U/ml) and penicillin (100 U/ml) under an atmosphere of 5% $CO_2$ and 95% humidified air at 37°C.

### Cell Viability Assay

Cell viability was determined by a modified MTT reduction assay. Cells were treated with MTT at 0.5 mg/ml. The purple formazan crystals were dissolved in DMSO. Solutions were then loaded in a 96-well plate, and determined on an automated microplate spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) at 570 nm.

### Serum Starvation

For Western blot analysis and NO assay, confluent HUVECs grown in 6-well and 12-well plates were serum-starved in phenol red-free HBSS (135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM KOH, 10 mM HEPES, 10 mM glucose, pH 7.4) supplemented with *L*-arginine (0.1 mM) for 30 min, followed by stimulation with various concentrations of Pic for 30 min or 1 h. This serum starvation was to prevent the deactivation of the normal enzyme activity by serum proteins, a procedure that is commonly used in the enzyme activity studies[26].

### Western Blot Analysis

Equal amounts of cytosolic extracts (20 mg) were electroblotted onto a nitrocellulose membrane, following separation using 8%-12% sodium dodecylsulfate-polyacrylamide gel electrophoresis. The blot was probed using the primary antibodies, and HRP-conjugated anti-IgG antibodies were used as the secondary antibodies to detect antibody-specific protein bands by WESTSAVE-Up® (AbFrontier, Seoul, Korea), a chemiluminescence detection kit. The band density that represents the phosphorylation and expression of eNOS and Akt protein were determined by Image J (image processing) software program (NIH, Bethesda, MD, USA) by a single investigator.

### NO Assay

NO production was determined by measuring the sum concentration of nitrite and nitrate in culture supernatants using a fluorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's protocol. Briefly, serum-starved HUVECs were treated with Pic

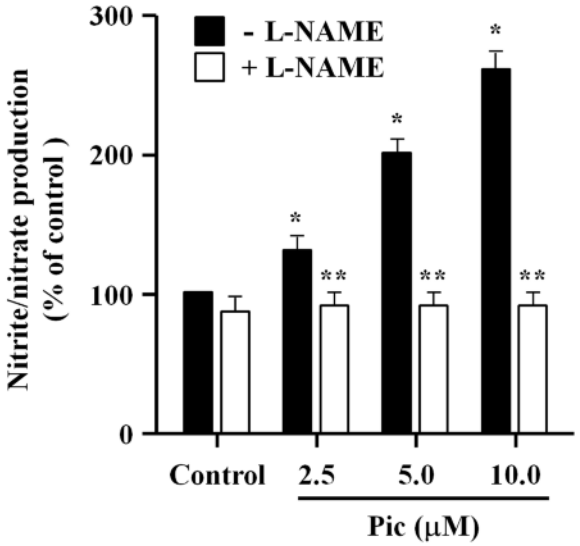


**Figure 1.** Concentration-dependent effects of Pic on NO production in HUVECs. Cells were pre-incubated for 1 h with medium or L-NAME (100 μM) and, then, exposed for 1 h to indicated concentrations of Pic, and the culture supernatants were then collected for NO assay. NO production was evaluated by measurement of nitrite and nitrate released to the culture media, as described in materials and methods. Data are expressed as mean ± SE from three separate experiments; $^{*}p < 0.05$ versus vesicle-treated control, $^{**}p < 0.05$ versus L-NAME-untreated group.

for 1 h or pretreated with LY294002 for 1 h before incubation with Pic. At the end of the incubations, culture supernatants were mixed with freshly prepared, light-protected 2,3-diaminonaphtalene (0.05 mg/ml in 0.62 M HCl) and incubated for 10 min at room temperature. The reaction was stopped with 2.8 M NaOH, and the fluorescent signal was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. The fluorescent signal was compared with the values given by nitrite standards (purity 98%) freshly prepared and dissolved in HBSS, normalized to protein concentration of the sample, and then expressed as percentages of vesicle-treated controls.

### *Statistical Analysis*

Results of all experiments are expressed as the mean ± standard error (SE) of multiple experiments ($n \geq 3$). Statistical analyses were performed using Student's two-tailed *t*-test or one-way ANOVA followed by Dunnett's multiple comparison with GraphPad Prism™ (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

## Results

### *Pic sStimulates Endothelial NO Production Through eNOS Phosphorylation*

To determine potential cytotoxicity of Pic, we treated the human endothelial cells, HUVECs, with different concentrations of Pic, and MTT assay for cell viability was performed after 24 h of incubation. Pic did not significantly influence the viability of HUVECs in the dose range between 1 and 10 mM (data not shown). At these non-cytotoxic concentrations, Pic increased nitrite/nitrate production in a concentration-dependent manner (Figure 1). To verify that the increase in nitrite/nitrate production in response to Pic specifically reflected NO release from eNOS, we examined the effects of the NOS inhibitor L-NAME that is capable of blocking eNOS activity and hence blocks NO release from eNOS. Nitrite/nitrate production by Pic treatment was abrogated by pretreatment of HUVECs with L-NAME (Figure 1), suggesting that an increase in nitrite/nitrate production after Pic treatment was due to NO release from increased eNOS activity. To determine whether NO production was attributable to eNOS activation that is linked to its phosphorylation state, we tested the effects of Pic on eNOS phosphorylation on residue Ser1177 by Western blot analysis. As shown in Figure 2, treatment of HUVECs with Pic resulted in a concentration-dependent increase in eNOS phosphorylation, with no significant change in total eNOS expression. Nitrite/nitrate production in response to Pic was rapid, being first observed at 30 min of Pic addition, and reached a maximum at 60 min, with no further increase in nitrite/nitrate production (Figure 3). In accordance with this, Pic-induced eNOS phosphorylation was first observed at 30 min, slightly reduced at 60 min, and almost completely decreased at 90 min (Figure 4). It was noteworthy that the levels of phosphorylated eNOS in response to Pic were similar to those of released NO. Thus, it was most likely that endothelial NO production in response to Pic was due to eNOS phosphorylation in HUVECs.

### *Pic Induces eNOS Phosphorylation Through Activation of PI3K/Akt*

It has been also reported that some polyphenolic compounds activate eNOS through PI3K/Akt pathway[27-32]. To elucidate one of possible upstream signaling pathways involved in Pic-mediated eNOS phosphorylation and

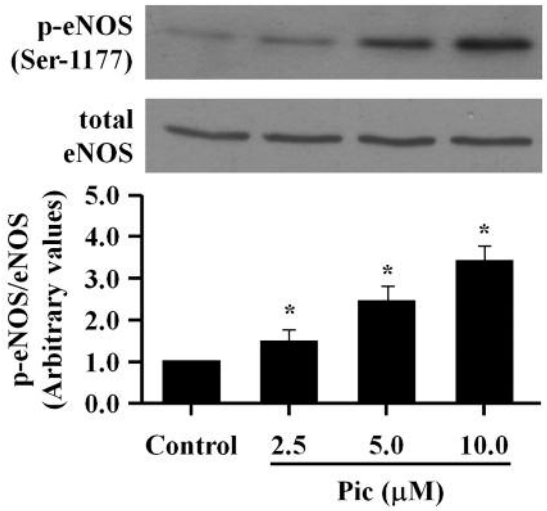


**Figure 2.** Concentration-dependent effect of Pic on eNOS phosphorylation in HUVECs. Cells were exposed for 30 min to indicated concentrations of Pic, and the total cell lysate was then subjected to immunoblotting with specific antibodies against p-eNOS (Ser-1177) and total eNOS. Relative changes in eNOS phosphorylation were assessed by scanning densitometry (upper panel). Representative blots are shown (lower panel). The experiment was repeated three times, and data are expressed as mean ± SE; *p < 0.05 versus vesicle-treated control.

subsequent NO release, we examined the activation of PI3K/Akt in Pic-treated HUVECs. Western blot analysis using an antibody specific to phosphor-Ser473 of Akt showed that Pic treatment caused a concentration-dependent phosphorylation of Akt (Figure 5). It was noteworthy that phosphorylation of Akt (Ser473) and eNOS (Ser1179) occurred in parallel. Based on such results, we next examined the effect of Pic on NO production in the presence of a PI3K/Akt inhibitor. As shown in Figure 6, endothelial NO production in response to Pic was significantly attenuated in the presence of the PI3K/Akt inhibitor LY294002. In line with this, eNOS phosphorylation in response to Pic was also reduced by LY294002 (Figure 7). These results, therefore, suggested that eNOS phosphorylation and subsequent NO release in response to Pic were likely to be mediated through PI3K/Akt signaling pathway. LY294002 itself has no effect on cell viability (data not shown).

## Discussion

Pic is believed to be a potent compound with cardiovascular therapeutic effects and also to have vasorelaxation and antioxidant activities[23]. However, the cellular and molecular mechanisms underlying the cardioprotective effects of Pic are still unclear. To our best knowledge, it is the first study to demonstrate that Pic, a natural hydroxylated analog of resveratrol, rapidly stimulates NO release from human endothelial cells.

The present work investigated whether rapid NO production by Pic could account for increased eNOS activity. The primary activity of eNOS is dependent on calcium-calmodulin activation. However, eNOS activity is also regulated at other levels, including protein-protein interactions and phosphorylation[5-7]. Among these, eNOS phosphorylation by some polyphenolic compounds appears to be the important means of regulating eNOS activity[6]. In the present research, we found that Pic, a polyphenolic compound, significantly induced a concentration- and time-dependent eNOS phosphorylation in HUVECs. Considering that eNOS phosphorylation is known to be one of therapeutic mechanisms for reversing endothelial dysfunction by enhancing the release of endothelial NO[33,34], our findings showing eNOS phospho-

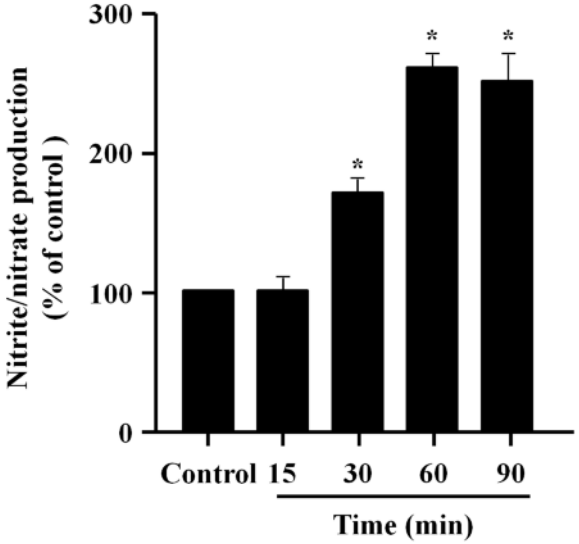


**Figure 3.** Time-dependent effect of Pic on NO production in HUVECs. Cells were exposed for indicated times to 10 μM Pic, and the culture supernatants were then collected for NO assay. NO production was evaluated by measurement of nitrite and nitrate released to the culture media, as described in materials and methods. Data are expressed as mean ± SE from three separate experiments; *$p < 0.05$ versus vesicle-treated control.

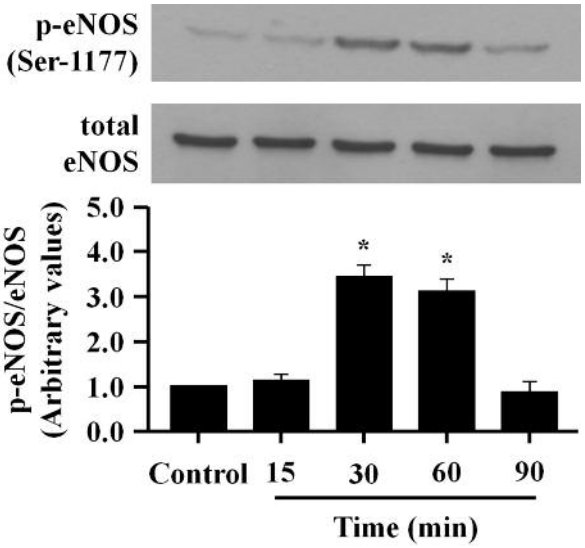


**Figure 4.** Time-dependent effect of Pic on eNOS phosphorylation in HUVECs. Cells were exposed for indicated times to 10 μM Pic, and the total cell lysate was then subjected to immunoblotting with specific antibodies against p-eNOS (Ser-1177) and total eNOS. Relative changes in eNOS phosphorylation were assessed by scanning densitometry (upper panel). Representative blots are shown (lower panel). The experiment was repeated three times, and data are expressed as mean ± SE; $^{*}p < 0.05$ versus vesicle-treated control.

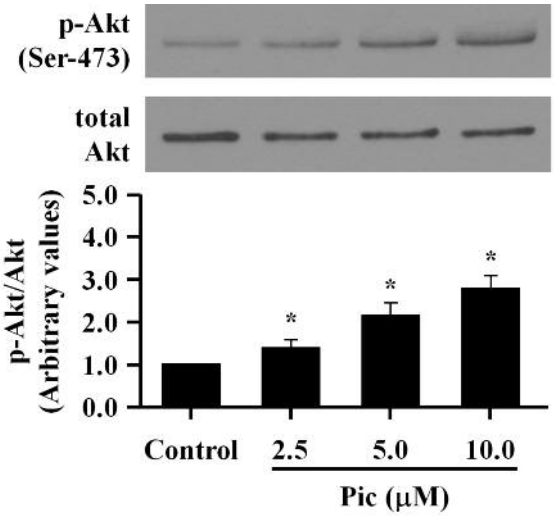


**Figure 5.** Effect of Pic on Akt phosphorylation in HUVECs. Cells were exposed for 30 min to indicated concentrations of Pic, and the total cell lysate was then subjected to immunoblotting with specific antibodies against p-Akt (Ser-473) and total Akt. Relative changes in Akt phosphorylation were assessed by scanning densitometry (upper panel). Representative blots are shown (lower panel). The experiment was repeated three times, and data are expressed as mean ± SE; $^{*}p < 0.05$ versus vesicle-treated control.

rylation by Pic in endothelial cells may in part explain its cardioprotective effects.

The present report also investigated whether eNOS phosphorylation by Pic could be mediated by the PI3K/Akt signaling pathway. The redox-sensitive PI3K has been shown to be activated by the redox sensitivity of the naturally occurring polyphenolic compounds[27-32]. Akt is a serine/threonine protein kinase that is recruited to the endothelial cell membrane because of its binding to PI3K-produced phosphoinositides. At the membrane, Akt is phosphorylated and induces eNOS phosphorylation, leading to NO production[35]. Studies performed in cell culture have also established that polyphenolic compounds affect the level of phosphorylation of Akt in a PI3K-dependent manner, which in turn stimulates eNOS phosphorylation, resulting in NO production[36]. In the present study, we observed that pretreatment of HUVECs with the PI3K/Akt inhibitor LY294002 significantly attenuated the NO production and eNOS phosphorylation induced by Pic. Moreover, we showed that Pic induced concentration-dependent Akt phosphorylation. Based on these data, eNOS phosphorylation

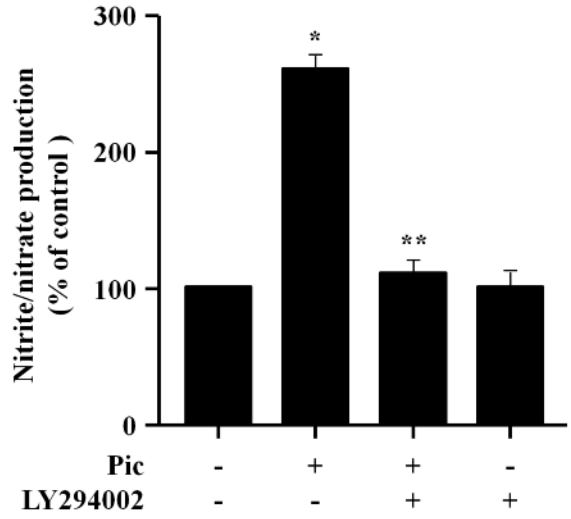


**Figure 6.** Effect of the PI3/Akt inhibitor LY294002 on Pic-induced NO production in HUVECs. Cells were pre-incubated for 1 h with medium or LY294002 (10 μM), and then exposed for 1 h to 10 μM Pic, and the culture supernatants were then collected for NO assay. NO production was evaluated by measurement of nitrite and nitrate released to the culture media, as described in materials and methods. Data are expressed as mean ± SE from three separate experiments; $^{*}p < 0.05$ versus vesicle-treated control, $^{**}p < 0.05$ versus Pic-treated group.

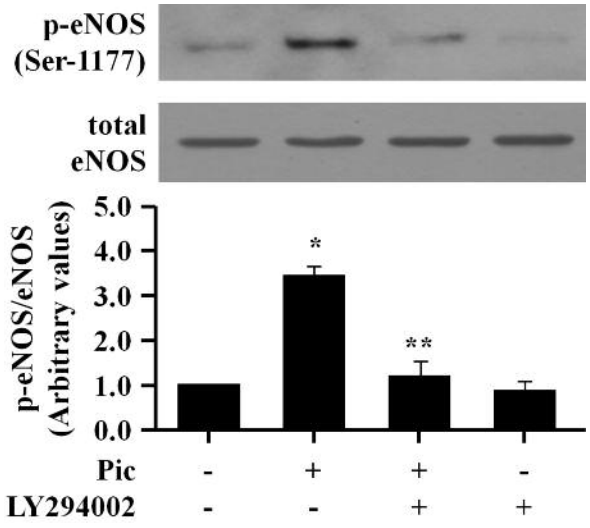


**Figure 7.** Effect of the PI3/Akt inhibitor LY294002 on Pic-induced eNOS phosphorylation in HUVECs. Cells were pre-incubated for 1 h with medium or LY294002 (10 μM), and then exposed for 30 min to 10 μM Pic, and the total cell lysate was then subjected to immunoblotting with specific antibodies against p-eNOS (Ser-1177) and total eNOS. Relative changes in eNOS phosphorylation were assessed by scanning densitometry (upper panel). Representative blots are shown (lower panel). The experiment was repeated three times, and data are expressed as mean ± SE; $^{*}p < 0.05$ versus vesicle-treated control, $^{**}p < 0.05$ versus Pic-treated group.

induced by Pic is likely to be mediated by activation of the PI3K/Akt signaling pathway. A similar mechanism of eNOS phosphorylation has been observed in response to the polyphenolic epigallocatechin-3-gallate[37] and polyphenol-rich products, such as a grape seed extract[38], grape skin extract[39], and strawberry powder[40]. Although it is obvious that PI3K/Akt is a key kinase in regulating eNOS phosphorylation, regulation of eNOS phosphorylation can be under the control of other kinases as well. Thus, it is important to design future studies to further determine the importance of other kinases on eNOS phosphorylation in response to Pic.

## Conclusions

This study, for the first time, demonstrates that Pic is capable of inducing a concentration- and time-dependent NO release *via* eNOS phosphorylation in endothelial cells. This effect is mainly mediated by activation of the PI3K/Akt signaling pathway, although the activation of other kinases cannot be ruled out. The potential efficacy of Pic could aid in the prevention of vascular diseases characterized by endothelial dysfunction. However, it is important to note that the effects of Pic described in this study were obtained with high concentrations that could not be measured in human plasma. It is suggested that a pharmacological intervention for Pic treatment could be necessary to reach higher plasma levels.

### Acknowledgements

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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